RNA UltraSense™ One-Step Quantitative RT-PCR System

Cat. No.: 11732-927 **Size: 100 reactions**

Store at -20°C

Description

The RNA UltraSense™ One-Step Quantitative RT-PCR System is a one-step quantitative RT-PCR system for the sensitive, reproducible detection of low-abundance RNA molecules using real-time detection instruments. This system combines the high-temperature reverse transcription capability of SuperScript™ III Reverse Transcriptase (RT) with the automatic hot-start PCR provided by Platinum® *Taq* DNA Polymerase in a highly concentrated formulation that is optimized for the detection of viral RNA and rare transcripts. Both cDNA synthesis and PCR are performed in a single tube using gene-specific primers and RNA. All components necessary for real-time RT-PCR are mixed in the tube, and reverse transcription is directly followed by PCR cycling without additional handling, enabling sensitive detection from as few as 10 copies of highly dilute RNA template.

The system consists of two major components: RNA UltraSense™ Enzyme Mix and RNA UltraSense™ 5X Reaction Mix. RNA UltraSense™ Enzyme Mix contains SuperScript™ III RT, Platinum® *Taq* DNA Polymerase, and RNaseOUT™ Ribonuclease Inhibitor. SuperScript™ III RT is a version of M-MLV RT that has been engineered to reduce RNase H activity and provide increased thermal stability (1, 2). The enzyme can be used to synthesize cDNA at a temperature range of 45–60°C, providing increased specificity, higher yields of cDNA, and more full-length product than other reverse transcriptases. SuperScript™ III RT is not significantly inhibited by ribosomal and transfer RNA, and can be used to synthesize cDNA from total RNA.

Platinum® *Taq* DNA polymerase is recombinant *Taq* DNA polymerase complexed with a proprietary antibody that blocks polymerase activity at ambient temperatures (3, 4, 5). Activity is restored after the denaturation step in PCR cycling at 94°C, providing an automatic "hot start" in PCR for increased sensitivity, specificity, and yield.

The RNA UltraSense™ 5X Reaction Mix consists of a proprietary buffer system, $MgSO_4$, dNTPs, and stabilizers. The 5X concentrated format allows you to add your low-abundance template in high volumes, and $MgSO_4$ is included in the mix at a final concentration that facilitates primer binding with difficult templates such as viral RNA. A tube of 50-mM $MgSO_4$ is included in the kit for further optimization of the Mg^{2+} concentration. A tube of 20X Bovine Serum Albumin (BSA) is included for compatibility with the Roche LightCycler®.

The RNA UltraSense™ One-Step Quantitative RT-PCR System has been formulated to provide optimal performance with Invitrogen's fluorogenic LUX™ Primers, and also supports fluorogenic hybridization probe-based detection methods such as TaqMan® probes (6–16). We do not recommend using this kit with dsDNA binding dyes such as SYBR® Green I. Sufficient reagents are provided for 100 amplification reactions of 50 µl each.

Note: This kit has been optimized for the rapid, convenient detection of low-abundance transcripts and viral RNA. For standard one-step quantitative RT-PCR, use the SuperScript™ III Platinum® One-Step Quantitative RT-PCR System (see **Additional Products**, below).

System Component	Amount
RNA UltraSense™ Enzyme Mix (includes RNaseOUT™ Ribonuclease Inhibitor)	250 µl
RNA UltraSense™ 5X Reaction Mix (contains 1 mM of each dNTP)	1 ml
20X Bovine Serum Albumin (BSA), UltraPure, non-acetylated (1 mg/ml)	300 µl
50-mM Magnesium Sulfate ($MgSO_4$)	1 ml
ROX Reference Dye	100 µl

Storage

Store components at -20ºC. Stability can be extended by storing at -80ºC. ROX Reference Dye must be stored in the dark.

Quality Control

The product is tested functionally by quantitative real-time analysis using total HeLa RNA as template. Kinetic analysis must demonstrate a linear dose response with decreasing target concentration and detection of β-actin mRNA in 1 pg of total HeLa RNA.

Additional Products

The following products are also available from Invitrogen.

Product	Amount	Catalog no.
SuperScript™ III Platinum® One-Step Quantitative RT-PCR System	100 rxns	11732-020
	500 rxns	11732-088
SuperScript™ III Platinum® Two-Step qRT-PCR Kit	100 PCRs	11734-050
	500 PCRs	11734-068
Micro-to-Midi Total RNA Purification System	50 rxns	12183-018
TRIzol® Reagent	100 ml	15596-026
	200 ml	15596-018
DNase I, Amplification Grade	100 units	18068-015
Custom Primers	to order, visit www.invitrogen.com	

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This product is distributed for laboratory research use only. CAUTION: Not for diagnostic use. The safety and efficacy of this product in diagnostic or other clinical uses has not been established.

For technical questions about this product, call the Invitrogen TECH-LINE℠ 800 955 6288

Recommendations and Guidelines for Quantitative Real-Time RT-PCR

RNA

RNA should be devoid of any RNase contamination and aseptic conditions should be maintained. RNaseOUT™ is included in the enzyme mix to safeguard against degradation of target RNA due to ribonuclease contamination of the RNA preparation.

To isolate total RNA or viral RNA, we recommend TRIzol® Reagent (Cat. Nos. 15596-026/-018). To isolate total RNA, you can also use the Micro-to-Midi Total RNA Purification System (Cat. no. 12183-018). Oligo(dT)-selection for poly(A)$^+$ RNA is typically not necessary, although incorporating this step may improve the yield of specific cDNAs.

Magnesium

The RNA UltraSense™ 5X Reaction Mix includes $MgSO_4$ at a concentration that facilitates primer binding with difficult templates such as viral RNA. If necessary, use the 50-mM magnesium sulfate solution included in the kit to increase the magnesium concentration.

Instrument Settings

This kit can be used with a variety of real-time instruments, including but not limited to the ABI PRISM® 7700/7000/7900 and GeneAmp® 5700, the Bio-Rad iCycler™, the Stratagene Mx4000™, Corbett Research's Rotor-Gene™, MJ Research's DNA Engine Opticon™, the Cepheid Smart Cycler®, and the Roche LightCycler®. Please refer to your instrument user manual for operating instructions. Optimal cycling conditions will vary with different machines. The protocols on the following page have been optimized for the ABI PRISM® 7700 and the Roche LightCycler®.

ROX Reference Dye

ROX Reference Dye included in each kit to normalize the fluorescent reporter signal in real-time RT-PCR. Its use is optional. ROX Reference Dye can be used to adjust for non-PCR-related fluctuations in fluorescence between reactions, and provides a stable baseline in multiplex reactions. It is composed of a glycine conjugate of 5-carboxy-X-rhodamine, succinimidyl ester (25 µM) in 20 mM Tris-HCl (pH 8.4), 0.1 mM EDTA, and 0.01% Tween® 20.

ROX is supplied at 50X concentration. Add 1 µl of ROX for every 50 µl of reaction volume.

Note that some TaqMan® probes utilize a TAMRA quencher, which can be used as a reference dye for normalization of data. This technique is only valid for a reaction containing a single TaqMan® probe, and should not be used in multiplex applications.

Bovine Serum Albumin (BSA)

Additional bovine serum albumin (BSA) is required in Roche LightCycler® reactions because the glass capillaries in the LightCycler® have a high surface-to-volume ratio and the glass surface binds molecules like *Taq* DNA polymerase. The addition of BSA blocks this surface binding.

Nonacetylated BSA is provided in the kit because acetylated BSA will inhibit PCR at the concentrations required in LightCycler® reactions. This inhibition is most likely due to the transfer of acetyl groups to essential components of the PCR, like the *Taq* DNA polymerase.

Primers

- Gene-specific primers (GSP) are required. We do not recommend using oligo(dT) or random primers, which may generate nonspecific products in the one-step procedure and reduce the amount of product.
- For mRNA, design primers that anneal to exons on both sides of an intron or within the exon/exon boundary to allow differentiation between amplification of cDNA and potential contaminating genomic DNA.
- Primers should be designed according to standard PCR guidelines. They should be specific for the target sequences, be free of internal secondary structure, and should avoid complementation at 3′ end within each primer, primer pair, or hybridization probe sequence (except as required for hairpin fluorogenic primers such as LUX™). For best results, the amplicon size should be limited to 80–200 bp.
- Optimal results may require a primer titration between 100 and 500 nM. A final concentration of 200 nM per primer is effective for most reactions.
- For multiplex applications, limit the amount of primer for the reference gene, such as β-actin or GAPDH, to avoid competition between amplification of the reference gene and sample gene. In general, the final concentration of the reference gene primer should be between 25 and 100 nM. However, a primer titration is recommended for optimal results.

Dual-Labeled Probes

- The optimal concentration of probe may vary between 50 and 800 nM. A recommended starting concentration is 100 nM.
- The probe sequence should be free of secondary structure and should not hybridize to itself or to primer 3′ ends.
- For multiplex applications, the concentration of each probe may need to be adjusted independently to obtain optimal fluorescent signals. The amount of probe for the reference gene, such as β-actin or GAPDH, should be limited as described above for primers.

Reaction Setup and Conditions

- Keep all components, reaction mixes and samples on ice. After reaction assembly, transfer them to a thermal cycler pre-heated to the desired cDNA synthesis temperature (45–60°C) and immediately start the RT-PCR amplification program.
- Efficient cDNA synthesis can be accomplished in a 15–30 min incubation at 45–60°C. Optimal temperature varies for different primers and templates. A good general starting point is 50°C for 15 min. For problematic templates, or to increase the specificity of cDNA priming, increase the cDNA synthesis temperature to 55°C.
- SuperScript™ III RT is inactivated, the RNA/cDNA hybrid is denatured, and Platinum® *Taq* DNA polymerase is activated during the 2-min incubation at 95°C.
- The annealing temperature should be 0–10°C below the melting temperature of the primers used.
- The extension time of 30 seconds for instruments that use PCR tubes/plates is appropriate for the short amplicons that are typically used in real-time PCR.

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Quantitative Real-Time One-Step RT-PCR Protocol

Separate cycling programs and reaction mixtures are provided for instruments that use PCR tubes/plates (e.g., ABI PRISM®, Bio-Rad iCycler™, Stratagene Mx4000™, Cepheid Smart Cycler®) and the Roche LightCycler®. A standard 50-µl reaction size is provided for instruments that use PCR tubes/plates; component volumes can be scaled as desired.

1. Program the real-time instrument to perform cDNA synthesis immediately followed by PCR amplification as shown below. Optimal temperatures and incubation times may vary for different target sequences (see **Reaction Setup and Conditions**, previous page).

Instruments using PCR tubes/plates	LightCycler®
50°C for 15 min hold*	*Program choice:* Amplification (**Note:** Detect fluorescence in the F1 channel.)
95°C for 2 min hold	*Analysis mode:* Quantification
40–50 cycles of:	45°C for 30 min hold
95°C, 15 s	95°C for 2 min hold
60°C, 30 s	50 cycles of:
	95ºC, 5 s
	55ºC, 10 s (single acquire)
	72ºC, 10 s
Melting Curve Analysis:	Melting Curve Analysis:
Refer to instrument documentation	*Program choice:* Melting curve
	Analysis mode: Melting curves
	95ºC, 0 s
	55ºC, 15 s
	95ºC, 0 s
	40ºC, 0 s

*42–50°C is acceptable. Use 50°C as a starting point.

2. Prepare a master mix on ice of all components except template, as specified below. **Note:** Preparation of a master mix is **crucial** in quantitative applications to reduce pipetting errors.

Reaction Component	Instruments using PCR tubes/plates: 1 rxn/100 rxns	LightCycler®: 1 rxn/34 rxns
RNA UltraSense™ Enzyme Mix [1]	2.5 µl/250 µl	1 µl/34 µl
RNA UltraSense™ 5X Reaction Mix	10 µl/1000 µl	4 µl/136 µl
20X Bovine Serum Albumin[2]	—	1 µl/34 µl
LUX™ Primers[3]		
Labeled primer, 10 µM	1 µl/100 µl	1 µl/34 µl
Unlabeled primer, 10 µM	1 µl/100 µl	1 µl/34 µl
or TaqMan® Probes		
Primer pair, 10 µM each	1 µl/100 µl	1 µl/34 µl
Fluorogenic probe, 10 µM	1 µl/100 µl	1 µl/34 µl
ROX Reference Dye (optional)	1 µl/100 µl	—
Total Master Mix Volume	15.5 µl/1550 µl	8 µl/272 µl

[1]Absence of genomic DNA in RNA preparations can be verified by omitting the SuperScript™ III RT/Platinum® *Taq* Mix and substituting 2 units of Platinum® *Taq* DNA polymerase in the reaction.
[2]Additional non-acetylated BSA is required for the LightCycler® reaction.
[3]In the LightCycler® reaction, the LUX™ fluorogenic primer must be FAM labeled.

3. Add the following to each reaction tube or well:

Component	Instruments using PCR tubes/plates	LightCycler®
Master mix	15.5 µl	8 µl
Template	up to 34.5 µl	up to 12 µl
DEPC-treated water	to 50 µl	to 20 µl

4. Cap or seal the reaction vessels, and gently mix to make sure that all components are at the bottom of the amplification tube. Centrifuge briefly if needed.
5. Place reactions in a preheated thermal cycler programmed as described above. Collect data and analyze results. After cycling, hold the reaction at 4°C until further analysis.

Troubleshooting Guide

Problem	Possible Cause	Probable Solution
No amplification product Relative fluorescent signal ≤ background or no template control	cDNA synthesis temperature too high, low priming efficiency	Lower incubation temperature.
	RT or cDNA primer blocked by secondary structure	Raise incubation temperature. Redesign primer(s).
	RNA has been damaged or degraded	Replace RNA if necessary.
	RNase contamination	Maintain aseptic conditions; add RNase inhibitor.
	Fluorescent probe not functional	Validate probe design and presence of fluorophore and quencher: Treat TaqMan® probe with DNase, and check for increase in fluorescence. Redesign and/or resynthesize probe if necessary.
Poor sensitivity	Not enough starting template RNA	Increase the concentration of template RNA; use 10 ng to 1 µg of total RNA.
Product detected at higher than expected cycle number	RNA has been damaged or degraded	Replace RNA if necessary.
	RNase contamination	Maintain aseptic conditions; add RNase inhibitor.
	RT inhibitors are present in RNA	Remove inhibitors in the RNA preparation by an additional 70% ethanol wash. Inhibitors of RT include SDS, EDTA, guanidium salts, formamide, sodium phosphate and spermidine (19, 20).
	Inefficient cDNA synthesis	Adjust cDNA synthesis temperature and/or primer design.
	Inefficient PCR amplification	Optimize PCR conditions: Adjust annealing temperature as necessary. Increase magnesium concentration. Redesign primers.
Higher than expected signal	Too much sample added to reactions	Decrease the concentration of template RNA.
Product detected at lower-than-expected cycle number, and/or positive signal from no-template controls	Template or PCR carry-over contamination	Isolate source of contamination and replace reagent(s). Use separate dedicated pipettors for reaction assembly and post-PCR analysis. Assemble reactions (except for target addition) in a DNA-free area. Use aerosol-resistant pipet tips or positive displacement pipettors.
Unexpected bands after electrophoresis	RNA contamination with genomic DNA	Pre-treat RNA with DNase I.
	Oligo(dT) or random primers used for first-strand synthesis	Use gene-specific primers.
	Low specificity in PCR	Optimize PCR conditions as described above.

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